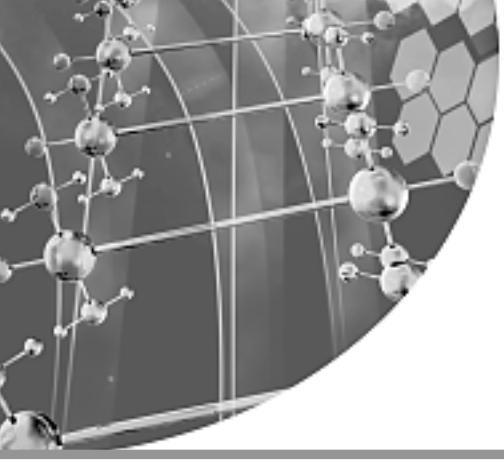

Manual Perfectprep™ BAC 96 Kit

For rapid and simple purification of
BAC, PAC, fosmid and cosmid DNA in
a 96-well format

Contents

Introduction

Bacterial Artificial Chromosomes (BACs) have become the vector of choice in the construction of libraries for genome sequencing projects due to their higher stability when compared to other large insert DNA vectors. BACs are based on the fertility (F) factor of *Escherichia coli*, which maintains strict copy number control, limiting the number of BACs to 1– 2 copies per cell. Limiting the copy number minimizes the risk of DNA recombination while maintaining a stable, cloned DNA insert within the host cell. However, due to the low number of BACs per cell, obtaining sufficient quantities of high quality BAC DNA when using lower reagent and starting material volumes, has posed a challenge for researchers. 5 PRIME has solved this problem with the development of the Perfectprep BAC 96 Kit which allows parallel processing of 96 samples.

The Perfectprep BAC 96 Kit is specifically designed for the purification of BAC DNA from *E. coli* bacterial cultures grown and processed in a 96-well plate format. Processing may be performed by using either a full vacuum or a centrifugation protocol. One 96-well plate can be processed manually in approximately 60 minutes and two plates in 60 – 75 minutes. The Perfectprep BAC 96 Kit is also compatible with isolation of other large circular constructs such as PACs, fosmids, and cosmids.

The versatility of the Perfectprep BAC 96 Kit enables easy integration onto automated liquid handling workstations such as the epMotion® 5075 Vac from Eppendorf or equivalent. The Perfectprep 96 BAC chemistry is also available in a high-throughput format (50 plate kit) for processing a large number of plates

The Perfectprep 96 BAC protocol uses a modified alkaline lysis procedure in which bacterial cells are lysed in the presence of RNase A. Using a proprietary technology, the liberated BAC DNA is trapped on a membrane surface, and then washed, resuspended, and eluted into a collection plate. The eluted BAC DNA is immediately ready for use in downstream applications. The Perfectprep BAC 96 Kit provides enough template for at least four BAC-end sequencing reactions and one fingerprint analysis.

The purified BAC DNA is suitable for use in the following downstream applications:

- Sequencing
- Fingerprinting
- Mutagenesis
- PCR

Precautions and warnings

Appropriate safety apparel such as lab coat, gloves, and eye protection should be worn. The following risk and safety phrases apply to components of the Perfectprep BAC 96 Kit:

- Solution 2
  Corrosive, contains: Sodium Hydroxide
  *R34, S26, S36/37/39, S45

- Solution 3
  Irritant
  *R36/38, S36/37/39

For more information, please consult the appropriate material safety data sheets which are available for this kit online at www.5Prime.com/msds.

*Risk and safety phrases:

R34 Causes burns;

R36/38 Irritating to eyes and skin.

S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice;

S36/37/39 Wear suitable protective clothing, gloves and eye/face protection;

S45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Kit Components Perfectprep BAC 96 Kit

Contents	Perfectprep BAC 96 Kit – 2 Plt	Perfectprep BAC 96 Kit – 10 Plt	Perfectprep BAC 96 Base Kit – 50[1]
Catalog number	**2300300**	**2300310**	**2300320**
Number of Samples Processed	**192**	**960**	**4800[1]**
Perfectprep BAC Solution 1	45 ml	300 ml	2 x 850 ml
Perfectprep BAC Solution 2	45 ml	300 ml	2 x 850 ml
Perfectprep BAC Solution 3	45 ml	300 ml	2 x 850 ml
RNase A Solution[2]	0.5 ml (10 mg/ml)	0.3 ml (100 mg/ml)	2 x 0.85 ml (100 mg/ml)
Lysozyme lyophilized[2]	40 mg	250 mg	2 x 625 mg
Perfectprep BAC Trapping Buffer Concentrate	12 ml	75 ml	2 x 250 ml
Perfectprep BAC Wash Buffer Concentrate	45 ml	2 x 150 ml	3 x 600 ml
Perfectprep BAC Elution Buffer	60 ml	260 ml	750 ml
Culture Plate	2	5 x 2	—
Filter Plate A	2	5 x 2	25 x 2
Perfectprep BAC Filter Plate BAC	2	5 x 2	25 x 2
Collection Plate	2	5 x 2	—
Plate Seals	25	3 x 25	3 x 25
Perfectprep BAC Air Permeable Seals	2	5 x 2	—

1without plates, please order Culture Plate, Catalog-No. 2300240, and Collection Plate, Catalog-No. 2300230, separately

2 please see pages 11 and 18 before starting the protocol

Additional Materials Required

For vacuum and centrifugation protocols:

- Plate shaker or vortexer (for cell resuspension)

Note: Any plate shaker or vortexer can be used, however, the Perfectprep 96 Bac protocols were optimized using the VWR Signature™ Multi-tube Vortexer (from VWR™) at setting 7, approximately 1,900 cycles per minute.

- Liquid bacterial growth medium and the appropriate antibiotic (see page 8f)
- 95–100% ethanol (for preparing the diluted Wash Buffer)
- 95–100% isopropanol (for preparing the diluted Trapping Buffer)
- Molecular Biology Grade Water (for use with the optional elution method, see page 16)
- Incubator/shaker at 37°C (for bacterial culture growth in 96-well culture plates)
- Centrifuge with microtiter plate rotor or deepwell plate rotor (for pelleting bacterial cultures, from Eppendorf or equivalent)
- 8 channel electronic 1200 µl pipette (from Eppendorf or equivalent)
- 8 or 12 channel, 10 –100 µl manual pipette (from Eppendorf or equivalent)
- Absorbent material (laboratory wipes, benchtop protector or paper towel)
- 200 additional plate seals for manual processing with the 50 plate kit only (see ordering information on page 30)
- Air Permeable Seals when using the 50 plate and 50 plate base kits (for growing bacterial cultures)

For vacuum protocol:

- Vacuum manifold for processing 96-well filter bottom plates using the vacuum protocol, such as 5 PRIME single basic manifold (see page 28)
  Note: The vacuum manifold must be compatible with the 96-well plates supplied in this kit. Manifold manufacturers other than 5 PRIME have not been tested.

For centrifugation protocol:

- 96-well catch plate capable of holding at least 2 ml (for use with centrifugation protocol), such as the 5 PRIME 2.4 ml Culture Plates (See ordering information on page 30)
- Centrifuge with a deepwell plate rotor, such as the Eppendorf centrifuges 5804 and 5810 and A-2-DWP Rotor or equivalent (for use with centrifugation protocol)

Storage and stability

Store all Perfectprep BAC 96 Kit components tightly sealed at room temperature. Do not freeze. All non-diluted Perfectprep BAC 96 Kit components are stable for at least 12 months when stored unopened as described above.

Quality assurance

Each lot of the Perfectprep BAC 96 Kit is functionally tested by isolating two different human BAC clones from *E.coli* cultures as described in the vacuum protocol. The purified BAC DNA is tested for quantity and quality by agarose gel electrophoresis and for performance in automated fluorescent cycle sequencing.

Bacterial cultures

Culture media

2x YT broth is the recommended culture medium for cultivating BAC clones with the Perfectprep BAC 96 Kit. This medium produces high cell densities and leads to excellent yields of low-copy number BAC DNA. Optimal growth conditions (24 hours at 37°C and 325 rpm) were determined using human BAC clones from the RPCI-11 library. Factors such as host strain and type of vector can drastically affect the yield of BAC DNA. Thus, growth conditions may need to be adjusted depending on the clones being purified. The appropriate antibiotic should be added to the medium, depending upon the antibiotic resistance gene present in the vector of the clones being grown. The recommended concentrations are 12.5 µg/ml of chloramphenicol for BACs and fosmids, and 25 µg/ml of kanamycin for PACs and cosmids.

Several other media have been tested using the optimal growth conditions for 2x YT, but all resulted in slightly lower DNA yields. If LB (Luria-Bertani) broth or 2x LB broth is used, the growth time may have to be adjusted to achieve the same DNA yields as 2x YT. If richer media such as Superbroth and Terrific Broth are used, the bacterial cells will grow to much higher cell densities compared to other media. However, this can lead to inefficient cell lysis and increased clogging when filtering the bacterial lysate. In addition, overgrowing these cultures can lead to a higher percentage of dead or starving cells in the culture and the DNA from these preparations may be degraded or contaminated with *E. coli* genomic DNA. Therefore, Superbroth and Terrific Broth are not recommended for use with the Perfectprep BAC 96 Kit.

Medium component formulations per liter

Culture Medium	Components	Components	Components
2x YT	16 g Tryptone	10 g Yeast extract	5 g NaCl
LB	10 g Tryptone	5 g Yeast extract	10 g NaCl
2x LB	20 g Tryptone	10 g Yeast extract	10 g NaCl

Inoculation of culture medium

The Perfectprep BAC 96 Kit was optimized for BAC DNA purification from cultures seeded directly from glycerol stocks, single colonies, or from pre-cultures of BAC clones. Each inoculation method has been tested and produced excellent results in downstream applications with the purified BAC DNA. Inoculations should be performed using a 96-pin replicator or multi-channel pipet using aseptic technique. Glycerol stocks should be stored at -80°C and should not be allowed to thaw for more than one hour. Repeated freeze/thaw cycles of glycerol stocks should also be avoided to maintain the integrity of the cells.

Growth and storage of bacterial cultures

1. Pipet 1.5 ml of 2x YT bacterial growth medium supplemented with the appropriate antibiotic into each well of a Perfectprep 96 BAC Culture Plate (2.4 ml deep well culture plate). See page 8f for information on antibiotics.

   2x YT medium is recommended for growth, but other media may be used with this kit (See page 8f).

2. Inoculate each well containing growth medium with a single BAC clone from glycerol stock or a single colony (See page 9).
3. Seal the plate using an Air Permeable Seal to protect against cross-well contamination while allowing the cultures to obtain sufficient aeration. Secure plates in an incubator/air shaker compatible with plate growth and incubate cultures for approximately 22– 26 hours at 37°C while shaking at 325 rpm.

Vacuum protocol

Before starting

- Please read through the entire protocol before proceeding.
- Check Perfectprep BAC Solution 2 for precipitate. If present, briefly warm solution at 37°C to re-dissolve.
- **Prepare the complete Perfectprep BAC Solution 1 (Perfectprep BAC Solution 1 plus RNase A Solution and lyophilized Lysozyme):**

1. Resuspend the lyophilized Lysozyme in the following amount of Perfectprep BAC Solution:

Kit Name	Perfectprep BAC 96 Kit – 2 Plts	Perfectprep BAC 96 Kit – 10 Plts	Perfectprep BAC 96 Base Kit – 50
Lysozyme lyophilized	40 mg	250 mg	2 x 625 mg
Add Perfectprep BAC Solution 1	1.8 ml	7 ml	2 x 14 ml

2. Mix thoroughly by pipetting up and down or gently reversing the tube. Take care to ensure that all of the powder is dissolved. Some foaming will occur.
3. Pipet the entire content of the Lysozyme Solution to the Perfectprep BAC Solution Mix thoroughly by pipetting up and down or gently reversing the tube before you proceed adding the RNase Solution to the Perfectprep BAC Solution 1.
4. Briefly centrifuge the RNase Solution to collect all liquid in the bottom of the tube.
5. Pipet the entire content of the RNase Solution to the Perfectprep BAC Solution 1 including Lysozyme. Mix thoroughly by pipetting up and down or gently reversing the tube.

- **Prepare appropriate amount of diluted Trapping Buffer:**

One plate: Combine 6 ml of the Trapping Buffer Concentrate and 18 ml of 95 –100% Isopropanol. Mix by inversion and store tightly sealed at room temperature.

Two plates: Combine 12 ml of Trapping Buffer Concentrate and 36 ml 95 –100% Isopropanol in a new bottle. Mix by inversion and store tightly sealed at room temperature.

→ **Prepare appropriate amount of diluted Wash Buffer:**

One plate: Combine 22.5 ml of Wash Buffer Concentrate and 52.5 ml of 95 –100% Ethanol in a new bottle. Mix by inversion and store tightly sealed at room temperature.

Two plates: Combine 45 ml of Wash Buffer Concentrate and 105 ml 95 –100% Ethanol in a new bottle. Mix by inversion and store tightly sealed at room temperature.

→ Prepare vacuum manifold:

Connect manifold to vacuum source using appropriate tubing. The vacuum source must be able to create a vacuum of approximately 70 kPa or 20 inches of mercury. Check to make sure that all valves are in the closed position and that bleed valves are in the open position.

Detailed vacuum protocol

1. Pellet bacteria by centrifuging the Culture Plate at 1,900 x g for 10 minutes.
2. After centrifugation, pour off supernatants and blot any remaining medium from the plate by inverting and tapping the plate on a clean absorbent material. Residual medium left in the wells can negatively affect the prep.
3. Pelleted bacterial cells can be used immediately, covered with a Plate Seal and stored at 4°C for up to four hours or -20°C for up to two months. It is recommended to use bacterial cells immediately.
4. Add 200 µl of Solution 1 to the cell pellets in each well of the Culture Plate. Cover plate securely using a Plate Seal.
5. Vortex the plate 2 –3 minutes on medium –high setting to resuspend the cell pellets. After vortexing for 2 minutes, check for complete dispersion of the pellet. If the cells are not clearly resuspended, the plate can be vortexed an additional minute.

→ Note: Complete resuspension of the pellets is critical to obtaining high yields of pure BAC DNA. Insufficient resuspension can result in downstream processing problems that could cause clogging of Filter Plate A in Step 14.

6. Add 200 µl of Solution 2 to each well of the Culture Plate to lyse cells. Cover plate securely using a Plate Seal.
7. Place the Culture Plate lid upside down over the Plate Seal. Apply pressure to the lid with both hands and mix the Culture Plate by inversion 5 times.

→ Caution: To prevent leakage and cross-well contamination, it is extremely important to keep constant pressure against the Plate Seal across the entire plate while mixing.

8. Incubate for 5 minutes at room temperature.
9. Add 200 µl of Solution 3 to each well of the Culture Plate to neutralize the bacterial lysate. Cover the Culture Plate securely using a Plate Seal.
10. Mix by inversion 10 times, as in step 7.
11. Prepare the manifold for the first vacuum step:

- Place a short adaptor (provided with the manifold) in the manifold chamber
- Position the Filter Plate BAC over the short adaptor
- Close the lid of the manifold
- Position the Filter Plate A on the manifold lid
- Be certain the plates are in the same orientation

12. Carefully peel off and discard the Plate Seal from the Culture Plate. Transfer contents (neutralized lysate) from the Culture Plate to the corresponding wells of the Filter Plate A. Set the pipette to 750 µl and transfer as much of the contents from the original Culture Plate as possible.

- Note: Pipetting lysate to the sides of the wells is recommended. Transfer of white cell debris to the Filter Plate A will occur and is not a problem. Save the used Culture Plate for a Waste Plate in Step 16. It may be necessary to move the pipet tip back and forth to collect as much lysate as possible.

13. When using the Single Vac or the Quad Vac manifold, turn on the vacuum pump and apply vacuum by switching vacuum valve to the open position and turning the bleed valve clockwise. It may be necessary to apply light pressure to the top of the plate by pressing down to engage the vacuum.
14. Vacuum until all liquid (neutralized lysate) flows through the Filter Plate A. Slowly bleed the vacuum by turning bleed valve counterclockwise. Allow the vacuum manifold chamber pressure to equalize to ambient pressure. Vacuum valve can now be switched to the closed position.

- Note: If some wells of the Filter Plate A clog, cover plate with a Plate Seal while the vacuum is being drawn. Covering the plate with a Plate Seal will increase the vacuum pressure. Some appearance of white flocculent material in wells is normal, and should not be mistaken for clogging. No more than 5 minutes should be spent in trying to get clogged wells to clear through the Filter Plate A.

15. Remove the Filter Plate A from the vacuum manifold and discard. Open the vacuum manifold lid and carefully remove the Filter Plate BAC and Collection Plate from the vacuum manifold chamber.

- Caution: Avoid contacting the bottom of the Filter Plate BAC with any surface during the plate transfers.

16. Place the used Culture Plate (from step 12) into the vacuum manifold chamber, close the vacuum manifold lid, and then position the Filter Plate BAC on the manifold lid.
17. Add 200 µl of diluted Trapping Buffer to each well of the cleared lysate in the Filter Plate BAC.
18. Cover the Filter Plate BAC securely using a Plate Seal. Place the Culture Plate lid upside down over the Plate Seal. Apply pressure to the lid with both hands and mix the Filter Plate BAC by inversion 4 times. Avoid touching drip directors.

→ Caution: To prevent leakage and cross-well contamination, it is extremely important to keep constant pressure against the Plate Seal across the entire plate while mixing.

19. Place the Filter Plate BAC on the manifold lid and incubate for 5 minutes at room temperature.
20. Carefully remove the Plate Seal from the Filter Plate BAC, then slowly apply vacuum (as described in step 13) until all liquid flows through the Filter Plate BAC. Bleed the vacuum (as described in step 14) and allow the vacuum manifold chamber pressure to equalize to ambient pressure.

    The BAC DNA molecules are now trapped on the surface of the membrane in the Filter Plate BAC.

21. Add 600 µl of diluted Wash Buffer to each well of the Filter Plate BAC. Slowly apply vacuum until all of the liquid has passed through the Filter Plate BAC. Bleed the vacuum and allow the vacuum manifold chamber pressure to equalize to ambient pressure.
22. Remove the Filter Plate BAC from the vacuum manifold and thoroughly blot the drip directors of the Filter Plate BAC several times on a clean absorbent material. Wipe excess liquid from around the edges of the bottom of the Filter Plate BAC with an absorbent material.

→ Note: Failure to blot the bottom of the Filter Plate BAC to remove excess liquid may result in unwanted reagent contamination of the BAC eluate. This could negatively affect the performance of the BAC DNA in downstream applications.

23. Open the vacuum manifold lid and remove the used Culture Plate from the vacuum manifold chamber and discard.
24. Place the Filter Plate BAC over the empty manifold chamber and allow it to air dry for 5 minutes.
25. Blot the drip directors of the Filter Plate BAC several times on a clean absorbent material to eliminate any ethanol remaining from the diluted Wash Buffer.

26. Place a Tall Adapter Plate (provided with manifold) inside the manifold chamber. Place an unused Collection Plate on top of a Tall Adapter Plate and close the manifold lid. Place the Filter Plate BAC containing the trapped BAC DNA on the manifold lid. Verify that both plates are in the same orientation.

→ Important Procedure Note:

Steps 27– 34 outline the standard elution method for this protocol. An optional elution method for increased yield and concentration is also available and is outlined on page 16. All downstream application procedures provided in this manual were performed using the standard elution method.

27. Add 30 µl of Elution Buffer to each well of the Filter Plate BAC. Add the Elution Buffer to the center of each well, just above the membrane.

→ Note: To avoid inconsistent elution volumes, ensure that the Elution Buffer is pipetted onto the surface of the filter and not the side of the well.

28. Incubate for 5 minutes.
29. Apply vacuum by turning bleed valve clockwise until completely closed. Vacuum until all liquid passes through the Filter Plate BAC.

→ Note: Liquid may appear to pass through the Filter Plate BAC membrane quickly; it is critical that vacuum pressure reach at least 15 inches of mercury before bleeding. Monitoring vacuum pressure will ensure that all liquid has passed through the Filter Plate BAC and into the Collection Plate.

30. Bleed vacuum and allow vacuum chamber pressure to equalize to ambient pressure.
31. Apply a second 30 µl aliquot of Elution Buffer to the center of each well, just above the membrane of the Filter Plate BAC.
32. Apply vacuum as in step 29 until the Elution Buffer has passed through the Filter Plate BAC. Slowly bleed the vacuum and allow the vacuum manifold chamber pressure to equalize to ambient pressure. Remove the Filter Plate BAC from the vacuum manifold and discard.
33. Open the vacuum manifold lid and carefully recover the Collection Plate from the vacuum manifold chamber.
34. DNA is immediately ready for use in downstream applications or may be stored. To store purified BAC DNA, cover the Collection Plate containing the purified BAC DNA using a Plate Seal and store at -20°C.

Optional elution method:

The following optional elution method can be used when increased yield and concentration are required. A dry down step is required following elution to reduce the final volume of the eluate and concentrate the DNA. Begin this procedure after step 26 of the vacuum protocol.

1. Add 60 µl of Elution Buffer to each well of the Filter Plate BAC. Add the Elution Buffer to the center of each well, just above the membrane.

→ Note: To avoid inconsistent elution volumes, ensure that the elution buffer is pipetted onto the surface of the filter and not the side of the well.

2. Incubate for 5 minutes.
3. Apply vacuum by turning bleed valve clockwise until completely closed. Vacuum until all liquid passes through the Filter Plate BAC.

→ Note: Liquid may appear to pass through the Filter Plate BAC membrane quickly; it is critical that vacuum pressure reach at least 15 inches of mercury before bleeding. Monitoring vacuum pressure will ensure that all liquid has passed through the membrane and into the Collection Plate.

4. Bleed vacuum and allow vacuum chamber pressure to equalize to ambient pressure.
5. Apply a second 60 µl aliquot of Elution Buffer to the center of each well, just above the membrane of the Filter Plate BAC. Apply vacuum as in step 3 until the Elution Buffer has passed through the Filter Plate BAC, then slowly bleed the vacuum and allow the vacuum manifold chamber pressure to equalize to ambient pressure.
6. Apply a third 60 µl aliquot of the Elution Buffer to the center of each well, just above the membrane of the Filter Plate BAC. Apply vacuum as in step 3 until the Elution Buffer has passed through the Filter Plate BAC. Slowly bleed the vacuum and allow the vacuum manifold chamber pressure to equalize to ambient pressure.
7. Dry BAC DNA samples in the Collection Plate for approximately 3 –3.5 hours at 60°C in a SpeedVac with a plate rotor or in a vacuum chamber. If a SpeedVac or vacuum chamber is not accessible, samples may be dried down overnight in an incubator set at 37°C.

→ Note: Prior to drying in a regular vacuum chamber or incubator the plate must be centrifuged briefly at 1,000 x g. Liquid should no longer be visible in the wells when the samples are completely dry.

8. Resuspend the samples in 30 µl of Molecular Biology Grade Water.
9. DNA is immediately ready for use in downstream applications or may be stored. To store purified BAC DNA, Cover the Collection Plate containing the purified BAC DNA using a Plate Seal and store at -20°C.

Centrifugation protocol

Before starting

- Please read through the entire protocol before proceeding.
- A centrifuge with a deep well, swing-bucket rotor capable of holding a 96-well filter plate on top of a deep well culture plate is necessary for this protocol (from Eppendorf or equivalent)
- Check Solution 2 for precipitate. If present, briefly warm solution at 37°C to re-dissolve.
- **Prepare the complete Perfectprep BAC Solution 1 (Perfectprep BAC Solution 1 plus RNAse A Solution and lyophilized Lysozyme):**

1. Resuspend the lyophilized Lysozyme in the following amount of Perfectprep BAC Solution:

Kit Name	Perfectprep BAC 96 Kit – 2 Plts	Perfectprep BAC 96 Kit – 10 Plts	Perfectprep BAC 96 Base Kit – 50
Lysozyme lyophilized	40 mg	250 mg	2 x 625 mg
Add Perfectprep BAC Solution 1	1.8 ml	7 ml	2 x 14 ml

2. Mix thoroughly by pipetting up and down or gently reversing the tube. Take care to ensure that all of the powder is dissolved. Some foaming will occur.
3. Pipet the entire content of the Lysozyme Solution to the Perfectprep BAC Solution Mix thoroughly by pipetting up and down or gently reversing the tube before you proceed adding the RNase Solution to the Perfectprep BAC Solution 1.
4. Briefly centrifuge the RNase Solution to collect all liquid in the bottom of the tube.
5. Pipet the entire content of the RNase Solution to the Perfectprep BAC Solution 1 including Lysozyme. Mix thoroughly by pipetting up and down or gently reversing the tube.

→ **Prepare appropriate amount of diluted Trapping Buffer:**

One plate: Combine 6 ml of the Trapping Buffer Concentrate and 18 ml of 95 –100% Isopropanol. Mix by inversion and store tightly sealed at room temperature.

Two plates: Combine 12 ml of Trapping Buffer Concentrate and 36 ml 95 –100% Isopropanol in a new bottle. Mix by inversion and store tightly sealed at room temperature.

→ **Prepare appropriate amount of diluted Wash Buffer:**

One plate: Combine 22.5 ml of Wash Buffer Concentrate and 52.5 ml of 95 –100% Ethanol in a new bottle. Mix by inversion and store tightly sealed at room temperature.

Two plates: Combine 45 ml of Wash Buffer Concentrate and 105 ml 95 –100% Ethanol in a new bottle. Mix by inversion and store tightly sealed at room temperature.

Detailed centrifugation protocol

1. Pellet bacteria by centrifuging the Culture Plate at 1,900 x g for 10 minutes.
2. After centrifugation, pour off supernatants and blot any remaining medium from the plate by inverting and tapping the plate on a clean absorbent material. Residual medium left in the wells can negatively affect the prep.
3. Pelleted bacterial cells can be used immediately, covered with a Plate Seal and stored at 4°C for up to four hours or -20°C for up to two months. It is recommended to use bacterial cells immediately.
4. Add 200 µl of Solution 1 to the cell pellets in each well of the Culture Plate. Cover securely using a Plate Seal.
5. Resuspend cell pellets by vortexing the plate for 2 –3 minutes on medium – high setting to resuspend the cells. After vortexing for 2 minutes, check for complete dispersion of the pellet. If the pellets are not clearly resuspended the plate can be vortexed an additional minute.

→ Note: Complete resuspension of the pellets is critical to obtaining high yields of pure BAC DNA. Insufficient resuspension can result in processing problems, such as clogging of the Filter Plate A in step 13.

6. Add 200 µl of Solution 2 to each well of the Culture Plate to lyse cells. Cover securely using a Plate Seal.

7. Place the Culture Plate lid upside down over the Plate Seal. Apply pressure to the lid with both hands and mix the Culture Plate by gently inverting 5 times.

→ Caution: While mixing, it is extremely important to keep constant pressure against the Plate Seal over the entire plate to prevent leakage and cross-well contamination.

8. Incubate for 5 minutes at room temperature.
9. Add 200 µl of Solution 3 to each well of the Culture Plate to neutralize the bacterial lysate. Cover the Culture Plate securely using a Plate Seal.
10. Mix by inversion 10 times, as in Step 7.
11. Place the Filter Plate A over a 96-well Catch Plate capable of holding at least 2 ml (not provided).
12. Carefully remove the Plate Seal from the Culture Plate and save the Culture Plate to be used later as a Waste Plate. Transfer contents (neutralized lysate) from the Culture Plate to the corresponding wells of the Filter Plate A. Transfer as much of the contents of the Culture Plate as possible by setting the pipette to 750 µl. Cover the Filter Plate A securely using a Plate Seal.

Pipetting lysate to the side of the wells is recommended. Transfer of white cell debris to the Filter Plate A is not a problem and will occur. Save the used Culture Plate for a Waste Plate in step 17.

13. Centrifuge the Filter Plate A/Catch Plate assembly at 1,900 x g for 2 minutes. Check to make sure all of the neutralized lysate has passed through the Filter Plate A. If lysate has not completely passed through the Filter Plate A, centrifuge at 1,900 x g for an additional 2 minutes. Discard the Filter Plate A after centrifugation.
14. Add 200 µl of diluted Trapping Buffer to each well of the Catch Plate containing the cleared lysate.
15. Cover the Catch Plate securely using a Plate Seal. Place a Culture Plate lid upside down over the Plate Seal. Apply pressure to the lid with both hands and mix by inversion 4 times.

→ Caution: While mixing, it is extremely important to keep constant pressure against the Plate Seal across the entire plate to prevent leakage and cross-well contamination.

16. Incubate for 5 minutes at room temperature.
17. Place the Filter Plate BAC over the Waste Plate (from Step 12). Transfer the Catch Plate contents to the corresponding wells of the Filter Plate BAC.
18. Centrifuge the Filter Plate BAC/Waste Plate assembly at 1,900 x g for 2 minutes. Check to make sure all of the cleared lysate has passed through the Filter Plate BAC. If not, centrifuge at 1,900 x g for an additional 2 minutes.

The BAC DNA molecules are now trapped on the surface of the membrane in the Filter Plate BAC.

19. Add 600 µl of diluted Wash Buffer to each well of the Filter Plate BAC.
20. Centrifuge the Filter Plate BAC/Waste Plate assembly at 1,900 x g for 2 minutes. Check to make sure all of the liquid has passed through the Filter Plate BAC. If not, centrifuge at 1,900 x g for an additional 2 minutes. Discard the flow-through from Waste Plate.
21. Blot the bottom of the Filter Plate BAC several times on a clean absorbent material. Wipe excess liquid from around the edges of the bottom of the Filter Plate BAC with an absorbent material.

→ Note: Failure to blot the bottom of the Filter Plate BAC to remove excess liquid may result in unwanted reagent contamination of the BAC eluate. This could negatively affect the performance of the BAC DNA in downstream applications.

22. Place the Filter Plate BAC over an unused Collection Plate. Add 30 µl of Elution Buffer to each well of the Filter Plate BAC. Add the Elution Buffer to the center of each well, just above the membrane.

→ Note: To avoid inconsistent elution volumes, ensure that the Elution Buffer is pipetted onto the surface of the filter and not the sides of the wells.

23. Incubate for 5 minutes at room temperature.
24. Centrifuge the Filter Plate BAC/Collection Plate assembly at 1,900 x g for 2 minutes.
25. Apply a second 30 µl aliquot of Elution Buffer to the center of each well, just above the membrane of the Filter Plate BAC.
26. Centrifuge the Filter Plate BAC/Collection Plate assembly at 1,900 x g for 2 minutes.
27. Remove the Filter Plate BAC/Collection Plate assembly from the centrifuge and discard the Filter Plate BAC.
28. DNA is immediately ready for use in downstream applications or may be stored. To store purified BAC DNA, cover Collection Plate containing the purified BAC DNA using a Plate Seal and store at -20°C.

Applications

Sequencing

BAC vectors are widely used in genome sequencing projects and provide the foundation for construction of BAC libraries used in genome mapping and sequencing. The Perfectprep BAC 96 Kit provides a fast and robust method for purifying BAC DNA to be used in applications such as DNA sequencing. The sequencing protocol below has been optimized for direct sequencing of BAC DNA on an ABI 3700 DNA sequencer. It incorporates the latest ABI BigDye™ advances and includes an improved reaction cleanup method, providing even better sequencing performance.

A standard 60 µl elution from the Perfectprep 96 Bac procedure provides enough BAC DNA for at least five sequencing reactions. Sequencing has been optimized using 1/4 BigDye Terminator v3.1 reactions in a 20 µl reaction volume. Excellent results have been observed using 10 µl of template from the standard elution method, independent of DNA concentration. Please see the Sequencing Data Section for examples of performance.

1. Add 5 –10 µl* of BAC DNA to a thermal cycling plate.
2. Denature the samples in a thermal cycler for 5 minutes at 95°C then place immediately on ice until the addition of the sequencing reaction master mix.
3. Prepare the sequencing reaction master mix as follows:

Reagent	Volume per reaction
BAC DNA Template	5 –10 µl*
5x Sequencing Buffer (ABI)	3 µl
BigDye Terminator Ready Reaction Premix v3.1 (ABI)	2 µl
Primer (10 µM)	1 µl
$MgCl_2$ (25 mM)	0.6 µl
H_2O	QS to 20 µl
Total Volume	20 µl

*10 µl of template is used when kit is processed using the standard elution method. 5 µl of template may be used when kit is processed using the optional elution method.

4. Add the appropriate volume of master mix to each sample so that the total reaction volume is 20 µl. Seal plate, mix well and centrifuge briefly.

5. Cycle the reactions according to the following program:
   a. 95°C for 5 minutes
   b. 95°C for 15 seconds
   c. 50 –55°C for 15 seconds
   d. 60°C for 4 minutes
   e. Repeat steps b – d for 100 cycles
   f. Hold at 4°C–10°C
6. Clean up sequencing reactions using the following protocol optimized for sequencing on the ABI 3700:

Before starting:

- A refrigerated centrifuge is required for this protocol. Verify that the temperature of the centrifuge is set at 4°C.
- Turn on the heat sealer for sealing plate before vortexing.
- Prepare 70% high purity ethanol and chill to -20°C.
- Note: BigDye Terminator chemistry is light sensitive. (A dark area, such as a drawer, should be available for the incubation steps.)

a. Add 5 µl of 125 mM EDTA to each well of the plate using a multi-channel pipet.

- Note: The EDTA must be directly added to the the reaction mix prior to proceeding to the next step. If necessary, gently tap the plate to ensure that the EDTA combines with the reaction mix.

b. Add 60 µl of 95 –100% high purity ethanol to each well of the plate using a multichannel pipet.
c. Seal the plate using a heat sealer and thoroughly vortex the plate for 8–10 seconds.
d. Incubate the plate at room temperature for 25 minutes in the dark.
e. Centrifuge the plate at 3,000 x g for 30 minutes at 4°C or 1,900 x g for 45 minutes at 4°C.
f. Pour off the supernatant into the sink with a flicking motion of the plate and then blot the plate on clean, absorbent material.
g. Invert the plate (unsealed) onto 2– 3 layers of clean absorbent material and centrifuge at 50 x g for 1 minute to remove any residual ethanol.
h. Add 150 µl of cold (-20°C) 70% high purity ethanol to each well of the plate using a multi-channel pipet and seal using a plate seal.
i. Centrifuge the plate at 1,900 x g for 15 minutes at 4°C.
j. Pour off the supernatant into the sink.
k. Invert the plate (unsealed) onto 2– 3 layers of clean absorbent material and centrifuge at 50 x g for 1 minute to remove any residual ethanol.
l. Air-dry the plate for 10 minutes in the dark. Seal the plate using a plate seal and store at -20°C until ready to sequence.

7. Resuspend the pellets in 8 µl of 0.1x TE immediately prior to sequencing.
8. Run the samples on an ABI 3700 DNA Sequencer using a 90 second injection time.

Sequencing data

The following data was generated using BAC clones from plate #707 of the CITB Human BAC library (Invitrogen/Research Genetics, Inc.). DNA from the 384 different clones was purified using the Perfectprep BAC 96 Kit according to the vacuum protocol and standard elution method described in this user manual. 10 µl of BAC DNA template was cycle sequenced using universal end-sequencing primer T7 (5'-TAA TAC GAC TCA CTA TAG GG-3'). Cycling, clean-up and sequencing were performed based on the protocols and parameters described in Section Sequencing on page 22. Quality scoring was performed using Phred base calling software (CodonCode Corporation). Passing scores were determined to be those samples with read lengths of greater than 100 Phred Q>20 bases.

Average sequencing data from BAC Clones

	Average Phred Q>20 bases	% Passing samples
Plate 1	673	93.8%
Plate 2	710	99%
Plate 3	702	93.8%
Plate 4	672	99%

Fingerprinting

BAC DNA fingerprinting uses restriction enzymes to digest the DNA and generate a number of DNA fragments that can be separated by agarose gel electrophoresis. Fingerprinting data from BAC clones are analyzed to determine which clones share a high percentage of the same size fragments, and therefore, the extent to which the clones overlap. From this, a contiguous series of overlapping clones is constructed, providing a detailed map to determine which BAC library clones are appropriate for sequencing. This is an effective method for organizing large genome sequencing project data.

BAC DNA purified using the Perfectprep BAC 96 Kit can be used immediately in fingerprinting reactions. We have found that 10 µl of Perfectprep BAC DNA can be completely digested with as little as 5 units of enzyme in a 20 µl reaction.

Troubleshooting

→ Poor cell growth

Comments	Suggestions
Use of wrong culture medium.	Use 2x YT medium to achieve the maximum yield with the growth conditions outlined in the protocol. If other media are used, the growth time may have to be optimized.
Use of wrong growth conditions.	Grow plates for 22–26 hours at 37°C and 325 rpm with proper aeration. Optimal growth times vary between clones due to differences in the host strain and vector, so test different times within the given range to determine what works best for the clones you are using.
Poor quality of glycerol stock.	Avoid repeated freeze/thaw cycles and do not let glycerol stocks remain thawed for more than 1 hour.

→ Filter Plate A clogs

Comments	Suggestions
Inadequate cell resuspension.	Ensure that cell pellets are completely resuspended in Solution 1 before lysing cells with Solution 2.
Un-equalized pressure applied across the plate during vacuum filtration.	Slowly apply the vacuum until it reaches the highest pressure and then apply a plate seal if some wells are still clogged.
Precipitated material from alkaline lysate clogging the filter membrane.	Apply the lysate to the sides of the wells of the Filter Plate A so that they are not directly applied to the membrane surface. Do not let the lysate sit on the Filter Plate A membrane for too long before applying the vacuum.
Frozen pellets were not completely thawed.	Verify that the cell pellets are completely thawed before adding Solution 1 to the culture plate.

→ Low DNA yields

Comments	Suggestions
Poor BAC DNA replication during host cell growth.	Some BAC clones do not replicate efficiently within their host cells. Even though the yields may be low, it is still possible to obtain adequate sequencing reads using the sequencing reaction conditions supplied in the protocol.
DNA molecules not resuspended in Elution Buffer prior to recovery by vacuum filtration or centrifugation.	Ensure that the Elution Buffer is applied directly to the Filter Plate BAC membrane surface and not to the sides of the wells. Make sure you incubate for 5 minutes.
Poorly grown cultures.	Use the culture media and growth conditions given in Section 7.
Frozen pellets were not completely thawed.	Verify that the cell pellets are completely thawed before adding Solution 1 to the culture plate.

→ Genomic DNA contamination

Comments	Suggestions
Excessive cell lysis after addition of Solution 2.	Mix gently by inverting the Culture Plate 5 times and do not exceed 5 minutes for the cell lysis incubation step. Minor *E. coli* genomic DNA contamination is possible but will not interfere with DNA sequencing.

→ Poor DNA sequencing

Comments	Suggestions
Sub-optimally grown cultures.	Proper cultivation of BAC clones is essential for obtaining sufficient quantities of high quality template DNA. Follow the inoculation and growth conditions outlined in Sections 7.1 – 7.3 of this user manual.
BAC template is difficult to sequence.	Redesign sequencing primers.
Excess BigDye terminator loaded onto sequencer.	Make sure sequencing reactions are properly cleaned up to purify the extension products before loading the samples on a DNA sequencer.

→ Poor DNA sequencing (cont.)

Comments	Suggestions
Ethanol contamination in the eluted DNA from not drying the Filter Plate BAC properly.	If the bottom of the Filter Plate BAC is not thoroughly dried according to Steps 21–24 in the Vacuum protocol, residual ethanol from the diluted Wash Buffer on the Filter Plate BAC may be carried over in the subsequent elution steps. If this occurs, the ethanol in the eluted DNA will inhibit cycle sequencing reactions, resulting in shorter sequencing read lengths. Make sure the drip directors of the Filter Plate BAC are thoroughly blotted on clean absorbent material and excess diluted Wash Buffer is wiped from the edges of the bottom of the Filter Plate BAC. Ensure that the Filter Plate BAC is air-dried for 5 minutes.
BAC DNA not sufficiently denatured before cycle sequencing.	Longer initial denaturing times are required for BACs due to the large length of the genomic DNA inserts. We have found that denaturing the template DNA for 5 minutes at 95°C prior to adding the sequencing reaction master mix, as described in Section Sequencing on page 22 of this user manual, yields longer sequencing read lengths.
Sub-optimal sequencing reaction mix conditions and cycle sequencing parameters.	Read lengths may vary for a variety of large-insert DNA clones depending on the host strain, type of vector, and the length of the genomic DNA insert. In addition, several factors such as the amount of template, the amount of primer, the reaction volume size, the length of the cycling extension step, and the number of cycles can all affect the sequencing read length results. Optimization experiments may be necessary to determine the ideal conditions for the clones being sequenced.

Additional information

5 PRIME vacuum manifolds

5 PRIME offers two single position vacuum manifold systems, Perfectprep Vac Manifold Single Basic and Perfectprep Vac Manifold Single. The manifolds can be used with a vacuum pump or a house vacuum if sufficient vacuum can be achieved. Normal atmospheric pressure is approximately 101 kPa or 30 inches of mercury. The vacuum source must be able to create a vacuum of approximately 70 kPa or 20 inches of mercury. Please see page 30 for ordering information.

Pressure conversions

To convert from kiloPascals (kPa) to	Multiply by
Millimeters of Mercury (mm Hg)	7.500638
Inches of Mercury (in Hg)	0.2953
Atmospheres (atm)	0.009869
Torrs (Torr)	7.500638
Millibars (mbars)	10
Pounds per square inch (psi)	0.145038

References

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5. Shizuya, H., Birren, B., Kim, U., Mancino, V., Slepak, T., Tachiiri, Y., and Simon, M. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc. Natl. Acad. Sci. 89: 8794 –8797.

Ordering information

Product	Package Size	Catalog No.
Perfectprep™ BAC 96 Kit	2 Plt	2300300
Perfectprep™ BAC 96 Kit	10 Plt	2300310
Perfectprep™ BAC 96 Base Kit	50 Plt[1]	2300320
Collection Plates	50 Plt	2300230
Culture Plates	50 Plt	2300240
Perfectprep™ BAC Solution 1	850 ml	2300330
Perfectprep™ BAC Solution 2	850 ml	2300340
Perfectprep™ BAC Solution 3	850 ml	2300350
Perfectprep™ Plasmid 96 Vac Kit	2 Plt	2300200
Perfectprep™ Plasmid 96 Vac Kit	10 Plt	2300210
Perfectprep™ Plasmid 96 Vac Base Kit	50 Plt[1]	2300220
Perfectprep™ Vac Solution 1	1000 ml	2300250
Perfectprep™ Vac Solution 2	1000 ml	2300260
Perfectprep™ Vac Solution 3	1000 ml	2300270
Perfectprep™ Vac Manifold	single basic	2300280
Perfectprep™ Vac Manifold	single	2300290

1without plates, please order Culture Plate, Catalog-No. 2300240, and Collection Plate, Catalog-No. 2300230, separately

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Perfectprep BAC 96 Kit quick centrifugation protocol

1. Inoculate 1.5 ml 2x YT medium
   Grow culture according to protocol

2. Centrifuge at 1,900 x g for 10 minutes to pellet cells

3. Pour off supernatant after centrifugation

4. Blot plate on absorbent material to remove residual culture medium

5. Add 200 µl Solution 1 to each well of Culture Plate
   Cover plate securely using a Plate Seal
   Resuspend pellets by vigorously vortexing the plate 2– 3 minutes

6. Add 200 µl Solution 2 to each well of Culture Plate
   Cover plate securely using a Plate Seal
   Place Culture Plate lid upside down over Plate Seal
   Apply pressure to the lid with both hands and mix by inversion 5 times
   Incubate samples for 5 minutes at room temperature

7. Add 200 µl Solution 3 to each well Cover plate securely using a Plate Seal
   Mix by inversion 10 times as before

8. Place Filter Plate A over a Catch Plate (not provided)
   Carefully remove Plate Seal from Culture Plate containing lysate
   Transfer contents to the corresponding wells of Filter Plate A

9. Centrifuge the Filter Plate A/Catch Plate assembly at 1900 x g for 2 minutes
   Discard Filter Plate A

10. Add 200 µl of diluted Trapping Buffer to each well of the Catch Plate
    Cover Catch Plate securely with a Plate Seal
    Place Culture Plate lid upside down over Plate Seal
    Apply pressure to the lid with both hands and mix by inversion 4 times
    Incubate for 5 minutes at room temperature

11. Place Filter Plate BAC over the Waste Plate (used Culture Plate)
    Transfer the Catch Plate contents to the corresponding wells of Filter Plate BAC

12. Centrifuge the Filter Plate BAC/Waste Plate assembly at 1,900 x g for 2 minutes

13. Add 600 µl diluted Wash Buffer to each well of Filter Plate BAC

14. Centrifuge the Filter Plate BAC/Waste Plate assembly at 1,900 x g for 2 minutes
    Discard Waste Plate
    Blot the bottom of the Filter Plate BAC several times on a clean absorbent material and wipe excess liquid from around the bottom edges of the Filter Plate BAC

15. Place the Filter Plate BAC over a Collection Plate
    Add 30 µl of Elution Buffer to the center of each Filter Plate BAC well
    Incubate 5 minutes at room temperature

16. Centrifuge the Filter Plate BAC/Collection Plate assembly at 1,900 x g for 2 minutes

17. Apply a second 30 µl aliquot of Elution Buffer to the center of each Filter Plate BAC well

18. Centrifuge the Filter Plate BAC/Collection Plate assembly at 1,900 x g for 2 minutes

19. DNA is ready to use in downstream applications

Perfectprep BAC 96 Kit quick vacuum protocol

1. Inoculate 1.5 ml 2x YT medium
   Grow culture according to protocol

2. Centrifuge at 1900 x g for 10 minutes to pellet cells

3. Pour off supernatant after centrifugation

4. Blot plate on absorbent material to remove residual culture medium

5. Add 200 µl Solution 1 to each well of Culture Plate
   Cover plate securely using a Plate Seal
   Resuspend pellets by vigorously vortexing the plate 2-3 minutes

6. Add 200 µl Solution 2 to each well of Culture Plate
   Cover plate securely using a Plate Seal
   Place Culture Plate lid upside down over Plate Seal
   Apply pressure to the lid with both hands and mix by inversion 5 times
   Incubate samples for 5 minutes at room temperature

7. Add 200 µl Solution 3 to each well to neutralize lysate
   Cover plate securely using a Plate Seal
   Mix by inversion 10 times as before

8. Prepare manifold for the first vacuum step:

- Place a short adaptor in the manifold chamber
- Position the Filter Plate BAC over the short adaptor
- Close the lid of the manifold
- Position the Filter Plate A on the manifold lid
- Be certain the plates are in the same orientation

9. Remove Plate Seal from the Culture Plate

10. Transfer Culture Plate contents to the corresponding Filter Plate A wells and set Culture Plate aside to be used later as Waste Plate

11. Apply vacuum by switching vacuum valve to the open position and turning bleed valve clockwise
    Vacuum until all liquid has passed through Filter Plate A
    Slowly bleed vacuum by turning bleed valve counter clockwise
    Allow the vacuum manifold chamber pressure to equalize to ambient pressure. Turn vacuum valve to the off position

12. Remove Filter Plate A from manifold and discard
    Remove Filter Plate BAC and Collection Plate from the manifold
    Place used Culture Plate into the vacuum manifold chamber and close manifold lid
    Place Filter Plate BAC on the manifold lid

13. Add 200 µl diluted Trapping Buffer to Filter Plate BAC
    Cover Filter Plate BAC securely with a Plate Seal
    Place the Culture Plate lid upside down over the Plate Seal
    Apply pressure to the lid with both hands and invert 4 times
    Avoid touching drip directors
    Place Filter Plate BAC on manifold lid
    Incubate for 5 minutes at room temperature

14. Carefully remove the Plate Seal from Filter Plate BAC
    Slowly apply vacuum as performed in previous vacuum steps
    When all liquid has passed through, bleed vacuum as before and allow chamber pressure to equalize to the outside

15. Add 600 µl diluted Wash Buffer to Filter Plate BAC Vacuum using above vacuum procedure

16. Remove Filter Plate BAC from the top of the vacuum manifold
    Thoroughly blot the bottom of the Filter Plate BAC several times on clean absorbent material
    Wipe excess liquid from bottom of Filter Plate BAC

17. Remove Culture Plate from the manifold and discard

18. Air dry Filter Plate BAC over the empty manifold chamber for 5 minutes
    Blot the bottom of the Filter Plate BAC several times on a clean absorbent material to eliminate residual diluted Wash Buffer
    Place a tall adapter in the manifold chamber
    Place a Collection Plate in the chamber on top of the adapter

19. Close the manifold lid
    Place Filter Plate BAC on top of the manifold in the same orientation as the Collection Plate

20. Add 30 µl of Elution Buffer to the center of each well of the Filter Plate BAC
    Incubate for 5 minutes at room temperature
    Vacuum liquid through the Filter Plate BAC using vacuum procedure previously described

21. Apply a second 30 µl aliquot of Elution Buffer to Filter Plate BAC

22. Vacuum as previously described

23. DNA is ready to use in downstream applications